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CUCURBITACIN B Δ^{23}-REDUCTASE FROM *CUCURBITA MAXIMA*

I. ASSAY METHODS, ISOLATION AND PURIFICATION*

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SUMMARY

1. Spectrophotometric and chromatographic assay methods were developed for cucurbitacin B Δ^{23}-reductase (NAD(P)H:cucurbitacin B Δ^{23}-oxidoreductase). One spectrophotometric method is based on the decrease in absorbance at 340 mμ in the presence of NADPH or NADH as electron donor while another method is based on the decrease in absorbance at 228 mμ as a result of the reduction of the Δ^{23}-bond in the side chain of cucurbitacin B.

2. The optimum pH of the enzyme was 6.65.

3. Final proof was obtained for the reduction of the Δ^{23}-bond in the side chain of cucurbitacin B.

4. Cucurbitacin B Δ^{23}-reductase was isolated from 7.1 kg of immature Green Hubbard fruits by extraction, centrifugation, alcohol fractionation, heat precipitation, pH precipitation, DEAE-cellulose chromatography and Sephadex G-75 gel filtration. The yield was 24 mg and the final enrichment 142.4.

5. The results of paper electrophoresis and ultracentrifugation indicated a homogeneous preparation with a sedimentation coefficient of 2.96 S.

6. Due to the fact that this enzyme falls in the class of the oxidoreductases with NADPH and NADH as the electron donors, it is proposed that the enzyme should be called NAD(P)H:cucurbitacin B Δ^{23}-oxidoreductase and it would be classifiable under the number EC 1.6.99.

INTRODUCTION

Cucurbitacin B Δ^{23}-reductase (NAD(P)H: cucurbitacin B Δ^{23}-oxidoreductase) is one of the enzymes involved in the breakdown pathway of toxic[1] bitter principles, the cucurbitacins, in plants. It catalyses one of the most important reactions in this

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pathway, *viz.* the reduction of the Δ^{23}-bond in the side chain of cucurbitacin B and possibly seven other cucurbitacins. These bitter principles occur in a wide variety of plants, and as a result of their toxicity[1] to man and animals, considerable importance is attached to studies of their chemistry and biochemistry. The cucurbitacin B Δ^{23}-reductase enzyme is of biochemical importance since its action is related to that of the side-chain reductases such as lanosterol reductase and desmosterol reductase which play an important role in the biosynthesis of cholesterol and some steroid hormones[2-6].

The final evidence and conclusions concerning the constitutional and stereochemical formulae of cucurbitacins A, B, C, D, E, F, I. J, K, and L were published recently by ENSLIN and co-workers[7-11]. The structural interrelationship of the different bitter principles are, therefore, well established. The structure of cucurbitacin B is given in Fig. 1.

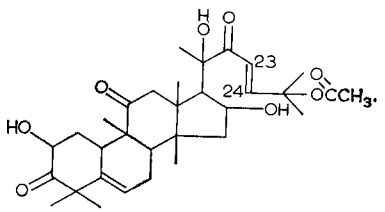


Fig. 1. Structure of cucurbitacin B.

Cucurbitacins B and E are the primary bitter principles formed in plants of the Cucurbitaceae family. The other bitter principles are most probably derivatives of cucurbitacins B and E produced as a result of metabolic processes which only take place during later stages of development[12,13].

It is postulated that the breakdown of cucurbitacins in plants starts with the deacetylation of the primary cucurbitacins by an acetyl-esterase enzyme[7,14]. This reaction is followed by enzymic reactions involving hydrogenation, dehydrogenation, hydroxylation, isomerisation as well as other unknown conversions. These reactions also occur *in vitro* during incubation of mixtures containing bitter principles and the juice of the fruits of the *Cucurbita maxima* species[15]. The products after incubation were detected by paper chromatography (refs. 12, 16 and 17; S. REHM, personal communication).

The fact that the 23,24-dihydrocucurbitacins were found to be better substrates for breakdown reactions *in vitro* than their precursor cucurbitacins with an unreduced side chain[12], underlines the important role of the cucurbitacin Δ^{23}-reductases in the breakdown pathway.

The purification of a reductase responsible for the reduction of the Δ^{23}-bond in the side chain of tetracyclic triterpenes has not yet been reported. Previous work on the cucurbitacin B Δ^{23}-reductase enzyme by TEIJEMA[12], yielded misleading results because reliable assay methods were not available. It was therefore decided to develop reliable assay methods for the reductase enzyme before attempting its purification.

MATERIALS AND METHODS

Materials

NADPH and NADH were obtained from Boehringer, Mannheim, Germany. Cucurbitacin B and 23,24-dihydrocucurbitacin B were kindly supplied by Dr. P. R. ENSLIN of the National Chemical Research Laboratories, C.S.I.R., Pretoria and Dr. S. REHM of the Horticultural Research Institute, Roodeplaat, Pretoria. DEAE-cellulose was obtained from Sigma, U.S.A. and Sephadex G-75 (Medium) from Pharmacia, Uppsala, Sweden. Green Hubbard fruits were obtained from the local market and from Mr. R. STARKE of Malelane, Transvaal. All other chemicals were of analytical grade quality.

Assay methods

A. Spectrophotometric assay at 340 mμ

The enzyme activity was determined by measuring the rate of decrease in absorbance of a reaction mixture containing NADH or NADPH, enzyme, substrate and metal co-factors at 340 mμ, employing the 'time drive attachment' of the Beckman model DK2A ratio recording spectrophotometer. An example of the composition of the reaction mixtures in the sample and reference cells is given in Table I.

TABLE I

COMPOSITION OF THE REACTION MIXTURES FOR SPECTROPHOTOMETRIC ASSAY AT 340 mμ

Maleic acid–NaOH buffer was used for the assay of the enzyme at a pH optimum of 6.65, because the metal co-factors were precipitated in phosphate buffers. Because of cucurbitacin B's low solubility in water, it was dissolved in abs. methanol. This solution was added last by injecting it very quickly with a micro-pipette into the sample cell. No precipitation occurred provided the concentration of cucurbitacin B was not in excess of 4.5 μmoles in 0.25 ml of abs. methanol (final methanol concn. in 3.0 ml reaction mixture, 8.3%, (v/v)). The NADPH was added just prior to cucurbitacin B. After the addition of cucurbitacin B and mixing, 30 sec elapsed before the absorbance measurement was started. In order to obtain a final pH value of 6.65, a maleic acid–NaOH buffer of pH 6.55 had to be used. All the measurements were carried out at 25° in the constant temperature cell holder of the Beckman model DK2A spectrophotometer.

Components	*Sample cell (ml)*	*Reference cell (ml)*
0.05 M maleic acid–NaOH buffer (pH 6.55)	2.05 − x	2.05 − x
0.015 M $MnCl_2$. Final Mn^{2+} concn. of 1 mM	0.20	0.20
Enzyme soln. in 0.05 M maleic acid–NaOH buffer (pH 6.6)	x	x
NADPH (or NADH), 0.56 μmole	0.50	0.50
Cucurbitacin B in abs. methanol, 3.5 μmoles	0.25	—
Abs. methanol	—	0.25
Total volume	3.00	3.00

At a final concn. of 8.3% (v/v) methanol, a reduction of 5% in the total activity compared with that in the presence of 5% methanol, was observed. The amount of substrate that could be dissolved in methanol concentrations below 5% (v/v) was too low to allow accurate activity measurements at zero-order kinetics. A methanol concentration of 8.3% (v/v) was found to be the most suitable.

One unit of reductase enzyme was defined as that amount which would catalyse the reduction of 1 μmole of cucurbitacin B or the oxidation of 1 μmole of NADPH

per min at 25° and optimum reaction conditions. In order to ensure zero-order kinetics, the concentration of enzyme selected had to be such that the maximum activity measurable at a cucurbitacin B concentration of 3.5 μmoles in 8.3% methanol in the presence of 0.56 μmoles NADPH, did not exceed $0.48 \cdot 10^{-5}$ to $0.58 \cdot 10^{-5}$ units. This activity range corresponds to a decrease of 0.05 to 0.06 in absorbance at 340 mμ in 5 min.

The μmoles of NADPH oxidized and thus μmoles cucurbitacin B reduced during the reaction were calculated from the molar absorbance coefficient of NADPH at 340 mμ which is given as $6.22 \cdot 10^6$ cm$^2 \cdot$mole^{-1} by HORECKER AND KORNBERG[18]. The velocity of the reaction in M$\cdot$sec^{-1} was also determined.

Blanks without enzyme showed no activity. Blanks without metal co-factors were completely inactive except when crude enzyme preparations, which were not dialysed or treated with EDTA, were used. When 23,24-dihydrocucurbitacin B was used as substrate, negligible activity was recorded in comparison with cucurbitacin B. This fact demonstrated very clearly that it was the Δ^{23}-bond which had undergone reduction.

The reductase activity obtained in the presence of Antimycin A or under anaerobic conditions in a N_2 atmosphere, did not differ from that obtained under aerobic conditions.

This method was used almost exclusively during the isolation, characterization and other studies[19] of the enzyme because of its accuracy and sensitivity.

B. Spectrophotometric assay at 228 mμ

The ultraviolet absorption spectrum of cucurbitacin B in methanol shows an absorption maximum at 228 mμ as a result of the α,β-unsaturated ketone grouping in the side chain. The molar absorbance coefficient of B at 228 mμ is $1.15 \cdot 10^4$ cm$^2 \cdot$ mole^{-1} (see refs. 20, 21). 2,3-Dihydrocucurbitacin B has no absorption maximum at 228 mμ. Consequently, the rate of reduction of cucurbitacin B (*i.e.* the reductase activity) can be determined very accurately by measuring the rate of decrease in absorbance at 228 mμ, and calculating the amount of conversion from the molar absorbance index of cucurbitacin B at 228 mμ.

The most important application of this method was to the unequivocal demonstration of the reduction of the Δ^{23}-bond in the side chain of cucurbitacin B. The reduction of the double bond in the side chain is the only transformation which can cause a decrease in absorbance at 228 mμ. If any of the other possible enzymic transformations of cucurbitacin B had taken place, *viz.* cucurbitacin B $\rightarrow$ D, B $\rightarrow$ C, B $\rightarrow$ A and B $\rightarrow$ E, no decrease in absorbance at 228 mμ would have occurred, since all of these products still have a α,β-unsaturated ketone grouping in the side chain.

The molar absorbance coefficient of cucurbitacin B at 228 mμ is much lower than that of NADPH or NADH at 340 mμ. It was therefore necessary to employ a much higher enzyme concentration as well as considerably longer reaction times to obtain a decrease in absorbance at 228 mμ of the same order of magnitude as that obtained in 10 min when the oxidation of NADPH was used as criterion. Thus, in the use of the spectrophotometric method at 228 mμ, compared with that at 340 mμ, under the same reaction conditions regarding metal co-factors and pH, the concentration of the enzyme should be increased 5 fold and the time of incubation from 10 min to 10 to 48 h. The theoretical decrease in absorbance which could be expected under these conditions was calculated from the reaction velocity obtained with the

TABLE II

CALCULATED DECREASE IN ABSORBANCE AT 228 mμ AS A RESULT OF CONVERSION OF CUCURBITACIN B TO 23,24-DIHYDROCUCURBITACIN B

Incubation time (h)	*Calculated decrease in absorbance*
10	0.056
24	0.14
48	0.28

spectrophotometric method at 340 mμ. The calculated values are given in Table II.

The composition of an incubation mixture for the spectrophotometric assay method at 228 mμ is given in Table III. This experiment was carried out at pH 7.5 with Zn^{2+} as metal co-factor.

In order to correct for chloroform-soluble substances which might show absorption in the 228-mμ region, a blank determination was performed on a reaction mixture similar to that given in Table III, except that cucurbitacin B was replaced with abs. methanol. The blank value thus obtained was subtracted from the value for the complete reaction to yield a corrected reaction value. This value was subtracted from the true blank value in order to obtain a final corrected value for the decrease in absorbance at 228 mμ due to the reduction of the Δ^{23}-bond in the side chain of cucurbitacin B. The true blank value can be defined as the blank determination when no enzyme was included in the incubation mixture. The composition of the true blank value reaction mixture is similar to that given in Table III except that the enzyme solution was replaced with 0.05 M Tris–HCl buffer (pH 7.5). This blank showed no decrease in absorbance at 228 mμ after an incubation time of as much as 50 h. The calculation of the final corrected decrease in absorbance at 228 mμ is summarized in the following relationship: Final corrected decrease in absorbance at

TABLE III

COMPOSITION OF AN INCUBATION MIXTURE FOR SPECTROPHOTOMETRIC ASSAY AT 228 mμ

After incubation at 30°, cucurbitacin B was extracted quantitatively from the incubation mixture with three 10-ml portions of chloroform. The combined chloroform extracts were evaporated to dryness in a constant temperature waterbath at 80°. The residue was dissolved in 6 ml of absolute methanol and the concentration of cucurbitacin B was determined spectrophotometrically at 228 mμ on a 3-ml portion of the methanol solution against an absolute methanol reference in a Beckman model DK2A ratio recording spectrophotometer. The chloroform and the methanol were distilled before use in order to remove impurities which absorbed at 228 mμ.

Component added	*Quantity (ml)*
0.10 M Tris–HCl buffer (pH 7.5)	1.175
Zinc acetate, final concn. 1 mM	0.30
NADPH, 2.5 μmoles	1.00
Enzyme soln. in 0.05 M Tris–HCl buffer (pH 7.5)	2.50
Cucurbitacin B in abs. methanol, 0.35 μmole	0.025
Total volume	5.000

228 mμ = true blank value — (value for complete reaction — blank value) = true blank value — corrected reaction value.

Determinations of reductase activity with this assay method were also performed at pH 6.65 in maleic acid–NaOH buffer at 30° with Mn^{2+} as the metal co-factor. A much lower enzyme concentration was necessary compared with the pH-7.5 determinations since Mn^{2+} was a much better metal co-factor at pH 6.65 than Zn^{2+}. A disadvantage of the determinations at pH 6.65 was that maleic acid was extracted in chloroform and caused high blank values as a result of the strong absorption of this acid in the 250-mμ region. The use of Antimycin A and anaerobic conditions had no advantage over aerobic conditions.

C. Paper and thin-layer chromatographic assay methods

The chromatographic methods[22] are useful when some rough measure of activity is desired and in detecting side reactions.

The incubation mixtures as well as the extraction procedures were similar to those described above except that a much higher concentration of cucurbitacin B was used (1.2 to 1.6 mg). About 400 to 800 μg of the extracted material was spotted on Whatman No. 1 chromatographic paper or cellulose chromatoplates. Development and detection were performed as described elsewhere[22].

Paper electrophoretic studies

High- and low-voltage paper electrophoresis were employed to gain more information about the isoelectric points of the various proteins in the solution obtained in purification step 4 and also as a measure of homogeneity of the preparation obtained in the final purification step.

Procion Blue R.S. was used as a staining reagent on the paper electrophoretograms[23].

Ultracentrifugal studies

The homogeneity of the purified reductase enzyme preparation was investigated in a Spinco model E ultracentrifuge. The sedimentation coefficient was determined by the sedimentation-velocity method[24]. A plain-window single-sector analytical cell was used in conjunction with the ultraviolet and Schlieren optical systems.

Determination of protein concentration

Protein concentration in eluates obtained after DEAE-cellulose and Sephadex G-75 chromatography were determined spectrophotometrically at 280 mμ. The nitrogen content was determined after each purification step by the micro-Kjeldahl method.

Isolation and purification of reductase enzyme

The enzyme was assayed spectrophotometrically at 340 mμ as described above at pH 6.65 in the presence of 1 mM $MnCl_2$. An arbitrary unit of enzyme activity was defined as the amount of enzyme which will produce a decrease of 0.001 in absorbance at 340 mμ in 5 min with the standard assay conditions. The specific activity is expressed as the number of arbitrary units per mg of protein. Due to the fact that the enzyme was found to be very unstable in the impure form, it was necessary to carry out the first purification steps in as short a time as possible.

Extraction Step 1. The edible part of fresh, skinned immature Green Hubbard fruits (total weight of 7100 g) was homogenized in the presence of 240 ml of 0.05 M maleic acid–NaOH buffer (pH 6.6), containing 1 mM EDTA, in a Automix blender for 30 sec. The homogenate was pressed through 8 layers of cheese cloth and centrifuged at 600 × *g* for 5 min. to yield 2300 ml of supernatant.

For assay purposes a small portion of the supernatant solution was dialysed overnight against 2 l of 0.05 M maleic acid–NaOH buffer (pH 6.6) at 1°, changing the buffer twice.

The supernatant was centrifuged at 44 000 × *g* for 30 min in a Spinco model L ultracentrifuge to yield a clear brownish-yellow supernatant (2230 ml). The sediment was discarded. A small portion of the supernatant was prepared for assay as above.

Alcohol fractionation. Step 2. Precooled ethyl alcohol (440 ml), at −20 to −30°, was added to the above supernatant solution over a period of 20 min until the alcohol concentration was 16.5% (v/v). The resulting precipitate was removed by centrifugation at 2000 × *g* for 10 min at −10°. The alcohol concentration of the supernatant was adjusted to 50% (v/v) and the precipitate collected as above. A third alcohol precipitate was obtained by the addition of another 1115 ml of precooled alcohol to the supernatant. Immediately after centrifugation, each of the three precipitates was suspended in 0.05 M maleic acid–NaOH buffer (pH 6.6), containing 1 mM EDTA (20, 110 and 35 ml, respectively) and dialysed overnight against 2 l of 0.05 M maleic acid–NaOH buffer (pH 6.6) changing the buffer solutions twice. After dialysis, the suspensions were centrifuged at 20 000 × *g* for 20 min and the sediments discarded. The supernatant solution of the 16.5–50% alcohol precipitate (297 ml) contained 93% of the total reductase activity of this step while the other two fractions contained only 6%.

Precipitation with $(NH_4)_2SO_4$ yielded unsatisfactory results. Acetone precipitation gave good results but the total yield of reductase activity was much lower than that obtained by alcohol precipitation.

Heat precipitation. Step 3. Heat precipitation was performed on the high-activity alcohol precipitate at 50° for 5 min. After the heat treatment the enzyme solution was immediately cooled in an ice-bath before centrifugation at 20 000 × *g* for 20 min. The sediment was discarded and the supernatant solution (288 ml) assayed for enzyme activity.

pH precipitation. Step 4. The pH of the solution obtained in Step 3 was adjusted to 4.7 by gradual addition of a 5% acetic acid solution (27 ml). The white precipitate was immediately removed by centrifugation at 20 000 × *g* for 20 min. The supernatant (316 ml) was decanted and the pH immediately adjusted to 6.6 by the addition of 5% NaOH (15 ml). This solution was dialysed overnight against 2 l of 0.01 M maleic acid–NaOH buffer (pH 6.6) with two buffer changes to yield 400 ml of diffusate. The precipitate contained no reductase activity. The diffusate was lyophilized for 13 h and the dry white powder thus obtained was dissolved in 23.5 ml of 0.05 M maleic acid–NaOH buffer (pH 6.6). The solution obtained was dialysed against 1 l of the same buffer with two buffer changes in 14 h to yield 32 ml of diffusate.

High- and low-voltage paper electrophoresis. The results of paper electrophoresis indicated that the enzyme solution obtained in Step 4 was very heterogeneous. Components with isoelectric points varying from below pH 4.9 to above pH 8.5 were

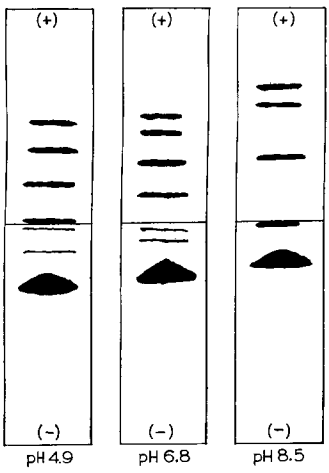


Fig. 2. High-voltage paper electrophoretograms of Step 4 enzyme preparation at 3 different pH values. Applications of 100 μl of concentrated protein solutions, containing 8.6 mg protein, were made to Whatman No. 1 paper previously impregnated with the appropriate buffer. The concentrated solutions were obtained by lyophilization. The high-voltage experiments were performed employing a voltage gradient of 137 V/cm at 7–8 mA, while the temperature was kept constant (6°). Running times varied between 2.5 and 3 h. Separate runs were made at pH values of 4.9, 6.8 and 8.5 in 0.01 M acetic acid–sodium acetate, maleic acid–NaOH and Tris–HCl buffers, respectively.

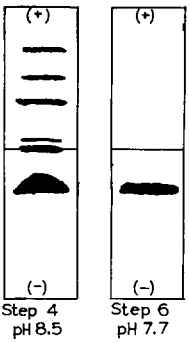


Fig. 3. Low-voltage paper electrophoretogram of Step 4 enzyme preparation at pH 8.5 and of Step 6 enzyme preparation at pH 7.7. After dialysis against 0.01 M maleic acid–NaOH buffer (pH 6.6), followed by lyophilization and, finally, dialysis against 0.05 M Tris–HCl buffers of pH 8.5 and 7.7, respectively, 4.3 mg protein of Step 4 and 2.5 mg of that of Step 6 were applied to Whatman No. 1 paper, previously impregnated with the appropriate buffer. A voltage gradient of 9.4 V/cm at 12–14 mA was employed at 2°. The running time was about 17 h.

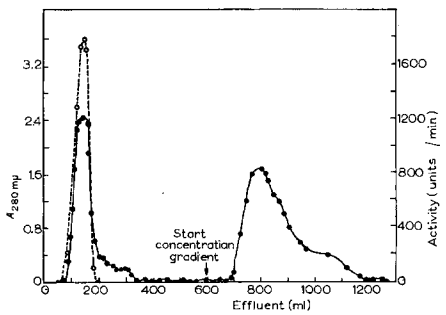
found. High-voltage paper electrophoresis yielded four anionic and three cationic groups of proteins as illustrated in Fig. 2.

The electrophoretograms from low-voltage electrophoresis of the Step 4 preparation as well as the purified enzyme preparation obtained in Step 6 are given in Fig. 3 and illustrate the cationic character of the enzyme at pH 7.7.

DEAE-cellulose chromatography. Step 5. A column of DEAE-cellulose (capacity 0.9 mequiv/g) 40 cm × 25 cm, was prepared and equilibrated with 1 l of 0.05 M maleic acid–NaOH buffer (pH 6.6). The enzyme preparation from Step 4 (30 ml) was applied to the column and, after 600 ml of 0.05 M maleic acid–NaOH buffer (pH 6.6) had passed through, a linear increase in molarity to 1.0 M over 800 ml at pH 6.6 was effected with 0.95 M NaCl in the usual manner. The elution pattern is illustrated in Fig. 4.

The first protein peak, which was eluted in 0.05 M maleic acid–NaOH buffer (pH 6.6), contained 39.6% of the total amount of protein applied to the column and all the reductase activity. The second peak contained 50.7% of the total protein but no reductase activity. Only 9.7% of the total protein could not be displaced with 1.0 M buffer.

The enzyme solution from the first peak was dialysed against 0.01 M maleic acid–NaOH buffer (pH 6.6) for 17 h, changing the buffer solution twice. The diffusate



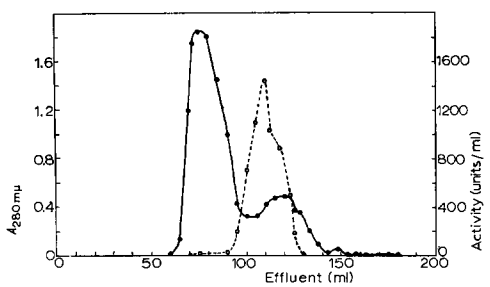


Fig. 4. Purification of cucurbitacin B Δ^{23}-reductase on DEAE-cellulose. The column was developed under a slight positive pressure at a flow rate of 35–40 ml/h. The effluent was collected in 5-ml fractions. Protein concentration was determined spectrophotometrically at 280 mμ against 0.05 M maleic acid–NaOH buffer (pH 6.6) in the reference cell of the Beckman model DK-2A spectrophotometer. The contents of tubes 17 to 35 were combined, since they contained the reductase enzyme (115 ml). ●—●, $A_{280\,m\mu}$; ○- - - - -○, reductase activity, units/ml.

Fig. 5. The purification of cucurbitacin B Δ^{23}-reductase on Sephadex G-75. The effluent was collected in 2.5 ml fractions. The protein concentration in the eluate was determined as described in the legend to Fig. 4. Fractions containing more than 180 units of reductase enzyme per ml were combined *i.e.* the contents of tubes 38 to 50, having a total volume of 28 ml. The remaining Step 5 solution was fractionated in a similar manner. ●—●, $A_{280\,m\mu}$; ○- - - - -○, reductase activity, units/ ml.

was concentrated by lyophilization to a volume of 10 ml. After the addition of 11.5 ml of 0.05 M maleic acid–NaOH buffer (pH 6.6), containing 0.10 M NaCl, the solution was dialysed overnight against the same buffer, changing the buffer solution twice. Reductase activity and protein were determined on the diffusate (23.75 ml).

Sephadex G-75 gel chromatography. Step 6. A column (115 cm × 1.5 cm) of Sephadex G-75 (Medium) was packed in 0.05 M maleic acid–NaOH buffer (pH 6.6) containing 0.10 M NaCl, under a 1 meter head of the same buffer. A portion of the enzyme preparation (11.2 ml) obtained in Step 5 was applied to the column and development was effected with the same buffer at a flow rate of 4 ml/h. The elution pattern is illustrated in Fig. 5.

The first peak eluted contained 82.5% of the total protein applied to the column and no reductase activity. The reductase enzyme was eluted just after the

TABLE IV

SUMMARY OF RESULTS OBTAINED IN THE COURSE OF THE ISOLATION OF CUCURBITACIN B Δ^{23}-REDUCTASE

Purification step	*Total protein (mg)*	*Total enzyme (units × 10^{-3})*	*Specific activity (units per mg protein)*	*Yield (%)*	*Purification factor*
1. Extraction	11 190	456.0	40.8	100.0	1.0
44 000 × *g* centrifugation	7 219	442.3	61.3	97.0	1.5
2. Alcohol precipitation	1 379	353.4	256.3	77.5	6.3
3. Heat precipitation	1 035	342.0	330.5	75.0	8.1
4. pH precipitation	454	247.2	543.9	54.2	13.4
5. DEAE-cellulose	175	148.2	847.6	32.4	20.8
6. Sephadex G-75	24	139.6	5812.0	30.6	142.4

first peak and the combined enzyme solution (28 ml) contained 13.7% of the total protein.

A final enrichment in reductase activity of 142.4 was obtained in Step 6 with a total yield of 30.6% (Table IV). The total amount of purified enzyme isolated from 7100 g of Green Hubbard fruits was 24 mg or 0.00034% of the total starting material.

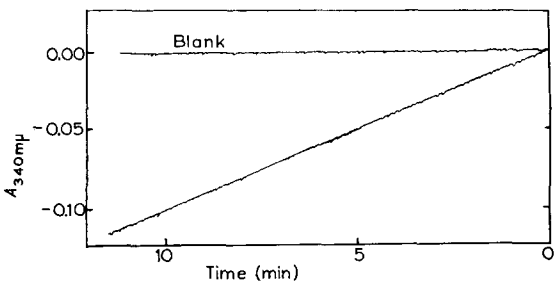


Fig. 6. Spectrophotometric assay of curbitacin B Δ^{23}-reductase at 340 mμ. The blank line represents the different blank determinations.

RESULTS AND DISCUSSION

Assay methods

An example of reductase activity determined at 340 mμ is illustrated in Fig. 6. In this case the calculated activity amounts to $0.48 \cdot 10^{-5}$ units while the reaction velocity is $2.68 \cdot 10^{-2}$ mμM$\cdot$sec^{-1}.

The results obtained with the spectrophotometric assay method at 228 mμ are given in Table V. The observed decreases in absorbance at 228 mμ are in good

TABLE V

RESULTS OF THE SPECTROPHOTOMETRIC ASSAY METHOD AT 228 mμ

The results tabulated below were obtained in experiments performed at pH 7.5 in Tris–HCl buffer and Zn^{2+} as metal co-factor, and at pH 6.65 in maleic acid–NaOH buffer with Mn^{2+} as co-factor.

pH and metal co-factor	*Incubation time (h)*	*Absorbance of cucurbitain B extracted from incubation mixtures*					
		Complete reaction value (A)	*Blank value (B)*	*True blank value (C)*	*Corr. reaction value (A − B)*	*Calc. theoretical decrease at 228 mμ**	*Obs. decrease at 228 mμ (C − A + B)*
pH 7.5, Zn^{2+}	10	1.43	0.046	1.43	1.384	0.056	0.046
pH 7.5, Zn^{2+}	24	1.46	0.12	1.46	1.34	0.14	0.12
pH 7.5, Zn^{2+}	48	1.28	0.12	1.48	1.16	0.28	0.32
pH 6.65, Mn^{2+}	48	1.14**	0.00**	1.44**	1.14	0.28	0.30

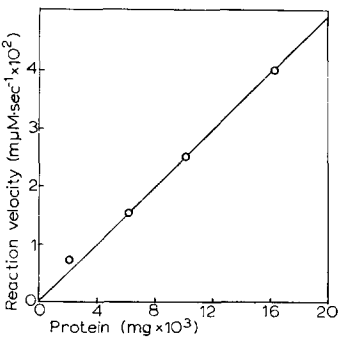
* Calculated from the reaction velocity obtained with the 340-mμ method under the same reaction conditions.

** A value of 1.276, due to the absorbance at 228 mμ of the maleic acid extracted from the incubation mixtures ,was found by means of a separate determination on a blank incubation mixture containing maleic acid–NaOH buffer, Mn^{2+} ,NADPH and methanol only. This value was subtracted from the complete reaction value, blank value and true blank value to obtain the correct values for A, B and C.

agreement with the calculated theoretical values. In the chromatographic assay methods two spots only, *viz.* those of cucurbitacin B and 23,24-dihydrocucurbitacin B were consistently observed. This finding together with the decrease in absorbance at 228 mμ provided final proof of the reduction of the Δ^{23}-bond. The 340 mμ method is undoubtedly the most sensitive, accurate and least troublesome assay method.

Effect of enzyme concentration on reductase activity

A linear relationship was found between reaction velocity and enzyme concentration as illustrated in Fig. 7.



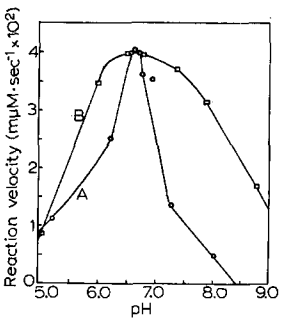


Fig. 7. Effect of enzyme concentration on cucurbitacin B Δ^{23}-reductase activity.

Fig. 8. Effect of pH on the activity of cucurbitacin B Δ^{23}-reductase. Curve A was plotted from results obtained with purified enzyme and Mn^{2+} and NADPH as co-factors, in the presence of maleic acid–NaOH and Tris–HCl buffers, over a pH range of 5 to 8. Curve B represents results obtained with crude enzyme and Zn^{2+} and NADPH as co-factors, in the presence of acetate and Tris–HCl buffers, over a pH range of 5 to 9.

pH optimum

The plots of reductase reaction velocity *vs.* pH, from results obtained under 2 different sets of conditions, are given in Fig. 8. In both cases the pH optimum was found to be 6.65 although the shapes of the curves are different. It appears as if in the presence of Zn^{2+}, the activity of crude enzyme preparations has a wider pH optimum range than purified enzyme in the presence of Mn^{2+}. It should be pointed out that a much lower concentration of enzyme was used for Curve A than for Curve B.

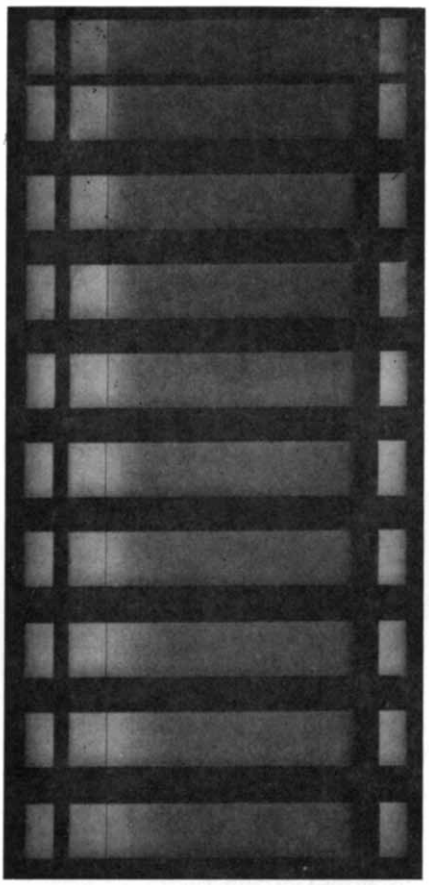
Stability of the enzyme

Although the enzyme was found to be very unstable in the impure form, the purified enzyme was fairly stable at room temperature and it retained activity for at least 5 weeks when stored at 0–4° in 0.05 M maleic acid–NaOH buffer (pH 6.6), containing 0.10 M NaCl. The enzyme was found to be relatively heat-stable. No loss of activity occurred when the enzyme solution was heated at 50° for 5 min but treatment at 50° for 10 min caused a loss of 30% of the total activity.

The determination of homogeneity by ultracentrifugation and paper electrophoresis

The purified enzyme preparation obtained in Step 6, was concentrated by lyophilization and dialysed against 0.15 M NaCl at a pH of approx. 6.5. This solution was used to determine the effect of protein concentration on the sedimentation coeffi-

cient at 4 different enzyme concentrations *viz.* 1.243, 0.829, 0.540 and 0.414 mg enzyme per ml. The ultraviolet optical system was employed. One determination with Schlieren optics was made at 1.243 mg protein per ml. An illustration of the moving boundary as obtained in a sedimentation velocity experiment employing the ultraviolet optical system is given in Fig. 9.



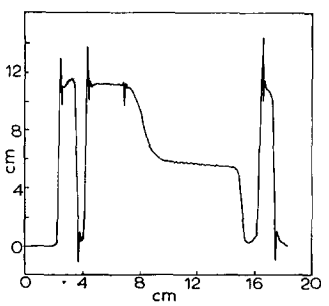


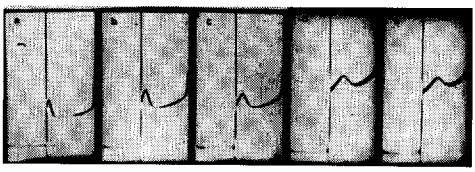
Fig. 9. An illustration of the moving boundary as obtained in a sedimentation-velocity experiment employing the ultraviolet optical system. A single sector analytical cell was used at 44 770 rev./min. The protein concentration was 1.243 mg/ml. Photographs were taken at time intervals of 8 min.

Fig. 10. Absorption diagram traced with a Spinco analytrol model R instrument. This diagram was obtained after centrifugation for 64 min at 44 770 rev./min. The enzyme concentration was 1.243 mg/ml.

An example of an absorption diagram traced with the use of a Spinco analytrol model R instrument is given in Fig. 10.

The ultracentrifugal patterns of the Schlieren experiment after different periods of centrifugation are given in Fig. 11.

Due to the limited amount of enzyme available, only one experiment, using the Schlieren optical system, could be performed.



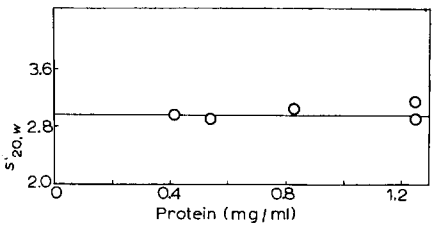


Fig. 11. Schlieren ultracentrifugal diagram of cucurbitacin B Δ^{23}-reductase. Photographs a, b, c, d and e were taken at 8, 16, 24, 48 and 56 min of centrifugation, after reaching 59 653 rev./min. Protein concentration was 1.243 mg/ml. A single sector analytical cell was used. Sedimentation was from left to right.

Fig. 12. The dependence of $s_{20,w}$ on enzyme concentration.

The sedimentation coefficient was found to be independent of enzyme concentration (c in mg/ml) with a final value of 2.96 S (Fig. 12).

The presence of a single sharp peak in Fig. 11 as well as the sharpness of the boundary illustrated in Fig. 10 indicated a homogeneous preparation. This was confirmed by the presence of a single component on low voltage electrophoretograms of the Step-6 enzyme preparation (Fig. 3). This component had a high electrophoretic mobility in the direction of the cathode and it corresponded to a large component found on other electrophoretograms of impure preparations. From these results it can be concluded that the isoelectric point of this protein must be higher than 8.5.

Classification of enzyme

Due to the fact that this enzyme falls in the class of the oxidoreductases with NADPH and NADH as electron donors, it is proposed that the enzyme should be called NAD(P)H: cucurbitacin B Δ^{23}-oxidoreductase, and it would be classifiable under the number EC 1.6.99 (see ref. 25).

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